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An amplified epidermal growth factor (EGF) receptor (EGF-R) cell line was compared with a non-amplified EGF-R variant of that cell ine. An EGF treatment time-course showed that the amplified cell line was unable to reduce its number of activatee EGF-R's as efficiently as the non-amplified cell line. This lack of negative regulation does not depend on covalent modification of the receptor by protein kinase C. However, the non-amplified variant line is negatively regulated by tyrosine phosphatases although the amplified cell line is not. Another difference between activated receptor negative regulation in these cell lines is that the variants can rapidly down-regulate activated receptors whereas the parental cell line cannot. Both differences may be due to limitations in the negative regulatory apparatus due to EGF-R amplification. In addition, another cell line that is not transformed but expresses an EGF-R compliment intermediate to the variant and the parental cell lines rapidly attenuates activated receptors with a rate similar to the non-amplified variants. The data supports the hypothesis that, amplification of the receptor in the absence of concomitant amplification of its negative regulatory components leads to dysregulated kinase activity and uncontrolled receptor signaling.

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Kevin Shaka 6/12/97

PI - Signature Date

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Introduction

The Role of EGF Receptor Negative Regulatory Components in Breast Cell Growth.

Amplification of the epidermal growth factor receptor (EGF-R) is correlated with tumorigenicity and its over-expression has been correlated with poor prognosis in breast cancer (1). EGF-R's bind ligand which causes phosphorylation of tyrosine residues on their C-terminal tail (2). This activation of the receptor allows it to signal to the cell via numerous interactions with other proteins. The EGF-R is negatively regulated both spatially and covalently. Spatially, receptor activation leads to rapid internalization, endocytic trafficking and targeting to lysomes for degradation, a process known as receptor down-regulation. Covalent regulation consists of phosphorylation of the receptor on serine and threonine residues which decrease the receptors affinity for ligand and decreases its kinase activity (2). Both forms of negative regulation control the overall extent of receptor signaling duration and intensity. Since amplification of the receptor is correlated with tumorigenicity, it was hypothesized that amplification of the receptor in the absence of concomitant amplification of its negative regulatory components would lead to dysregulated kinase activity and uncontrolled receptor signaling. Because EGF-R kinase activity determines both its mitogenic and transforming ability, dysregulation would lead to uncontrolled growth and contribute to the formation of breast cancers (3). This hypothesis is being tested using cell culture models.

Statement of Work

Part I.

Determine the percent reduction of EGF-R kinase activity following PMA or EGF induced desensitization for all cell types being used.

Months 1-12

Body of Report

The primary cell culture system used in this work is based on the malignant breast cell line MDA-MB-468 (4). Filmus *et al.* selected variant clones of the parental 468 line that had lost genomic amplification of the EGF-R locus and thus had reduced EGF-R levels (5). The parental cell line has 1.9 x10^6 EGF-R's per cell, the S1 variant 1.6x10^4 EGF-R's per cell and the S11 variant has 6.6x10^4 receptors per cell. The parental cell line has a faster growth rate and forms larger, more aggressive tumors in nude mice than either of the variants (S1 or S11) (5). Therefore, the tumorigenicity of these cells is directly correlated to their EGF-R expression level.

A second cell culture system was also used for this report. The A1 cell line is a human mammary epithelial cell isolated by reduction mammoplasty and immortalized by chemical mutagenesis (6, 7, 8). The A1 cell line, though immortal, does not exhibit anchorage independent growth, does not form tumors in nude mice, is absolutely dependent on EGF (epidermal growth factor) growth, and expresses 2x10^5 EGF-R per cell (6, 7, 8). For these reasons, this cell type is used because it has an intermediate receptor number and is functionally "normal".

An ELISA was performed in order to determine the duration of EGF-R signaling following treatment with EGF. Figure 1 shows the result of the ratiometric determination of PY/EGFR (phosphotyrosine per EGF-R) for the parental cell line (468), and each of the variants (S1 and S11). PY/EGFR is a measure of the relative number of activated EGF-R's. Cells were treated for 0, 2, 30, or 60 minutes with 100 ng/ml EGF. Protein was extracted and PY/EGFR was determined by ELISA. These results show that the amplified EGF-R parental cell line (468) maintains a maximum number of activated receptors from 2 minutes through 1 hour. In contrast, both of the variant cell types (S1 and S11), that have lost their EGF-R amplification, show a significant reduction in the number of activated receptors from 2 minutes through 1 hour. These results show that the non-amplified receptor variants are able to rapidly attenuate EGF-R signaling while the amplified receptor subtype (468) cannot.

An immunoprecipitation was performed in order to confirm the results in figure 1 by doing the experiment in a slightly different manner. The cells were treated for 0, 10, 30, 60 or 120

minutes with 100 ng/ml EGF. Protein was extracted, the EGF-R's were immunoprecipitated with an anti-EGFR antibody and probed on a western blot for phosphotyrosine (PY). Figure 2 shows that the total level of PY decreases significantly in each of the non-amplified variants (S1 and S11) from a maximum level at 10 minutes post-EGF treatment to a minimum level at 1 hour post-EGF treatment. However in the amplified parental cell line (468), maximum levels of PY achieved at 10 minutes post-EGF treatment are maintained after 120 minutes post-EGF treatment. As in figure 1, these results show that the number of activated EGF-R's is rapidly attenuated following EGF treatment in the non-amplified variants whereas the 468 parental cell line maintains a high level of activated receptors even after 2 hours.

Several negative regulatory pathways could be responsible for the results shown in figure 1 and figure 2. First, the EGF-R's could be rapidly desensitized by covalent modification in the variants but the high level of receptors in the parental cell line may prevent efficient desensitization of those receptors. Second, tyrosine phosphatases could be effective in the non-amplified variants but become limiting, and thus ineffective, in the amplified receptor parental cell line. Lastly, down-regulation of the small number of EGF-R in the non-amplified variant cell types could be an effective mechanism of rapidly attenuating EGF-R signaling. In this case, the amplified receptor parental cell line may not be effectively attenuated because the large numbers of receptors may be saturating the endocytic machinery.

Since phosphorylation of the EGF-R residue threonine 654 (T-654) by protein kinase C (PKC) is a well characterized method of desensitizing the receptor, the cells were treated with 1.6 uM PMA for 0, 10, 30 or 60 minutes (figure 3). Following PMA treatment the cells were treated for 10 minutes with 100 ng/ml EGF. The results in figure 3 show that the same maximum level of PY is attained with 10 minutes EGF treatment regardless of the duration of PMA treatment. This data indicates that, at least in 468 cells and its variants, desensitization of the receptor due to PKC phosphorylation of T-654 is a minor or unimportant mechanism of negative regulation.

The second explanation for the results obtained for figure 1 and figure 2 is that the EGF-R's in the variant cell lines are rapidly dephosphorylated by tyrosine phosphatases while the high

level of receptors in the parental cell line prevent efficient dephosphorylation by phosphatases. This hypothesis was tested by treating the cells with EGF or with EGF and orthovanadate (OV), a tyrosine phosphatase inhibitor, simultaneously (figure 4). Cells were treated with 100 ng/ml EGF and with or without 1 mM OV for 0, 10, 30, 60 or 120 minutes. After incubation, the cellular proteins were isolated and the EGF-R's were immunoprecipitated with anti-EGF-R antibodies. PY levels were quantified by western blotting with an anti-PY antibody and all values were normalized to 100% PY activity at 10 minutes post EGF treatment. The parental amplified receptor cell line (468) does not appear to have increased maximal PY levels regardless of the duration of OV treatment. However, treatment of the non-amplified variant (S1) with orthovanadate significantly prolongs the maximum level of receptor PY these cells have overtime. These results show that phosphatases are an important negative regulatory control in the non-amplified variants but they do not seem to have a significant effect on the amplified parental cell population. This suggests that at least part of the reason that the level of activated receptors remains elevated over time in the amplified receptor cell line (468) is because the tyrosine phosphatases are ineffective at dephosphorylating such a large number of receptors.

In order to determine if cell lines over-expressing EGF-R's are impaired in their ability to efficiently down-regulate the receptor, an EGF-R immunoprecipitation was performed after treating the cells for 0 or 120 minutes with 100 ng/ml EGF (figure 5). After EGF treatment, the cells were lysed and proteins were extracted. The EGF-R's were immunoprecipitated with anti-EGF-R antibodies and western blotted with a different anti-EGFR antibody. The results show that the amplified receptor parental cell line (468) is not significantly down-regulated after 120 minutes. However, both of the non-amplified receptor variants (S1 and S11) have reduced their total receptor mass to less than 50% of its initial value after 120 minutes. This result indicates that in addition to a reduction in phosphatase activity (figure 4), the amplified receptor cell line is unable to efficiently down-regulate such a large number of receptors which would contribute significantly to its inability to reduce its large number of activated EGF-R's.

Another cell line, A1, was used to further test the hypothesis because it is phenotypicaly "normal" and it has a receptor number intermediate to the non-amplified variant cell lines (S1 and S11) and the amplified receptor parental cell line (468). The hypothesis predicts that because the A1 cells are not transformed, even though they have a large number of receptors they should also have the negative regulatory capacity for that number of receptors. Figure 6 shows a comparison of A1 and 468 cell types PY. Cells were treated from 0 to 6 hours with 100 ng/ml EGF. After incubation the cellular proteins were isolated and the EGF-R was immunoprecipitated with anti-EGF-R antibodies. The bands were then quantified by western blotting using an anti-PY antibody. The figure shows that the level of PY is only slightly reduced in the 468 cell line after 6 hours EGF treatment. However, the PY levels in the A1 cells is rapidly reduced after EGF treatment and has returned to nearly pre-EGF treatment levels after 6 hours. These results support the hypothesis that amplification of the receptor in the absence of concomitant amplification of its negative regulatory components would lead to dysregulated kinase activity and uncontrolled receptor signaling.

The results from figure 3 show that desensitization of the receptor by phosphorylation at T-654 is not an important negative regulatory control in the 468 cell line and its variants. For this reason, the transfection of A-654 and S-654 mutants will not be performed with these cells. Instead, only wildtype tagged receptors will be transfected into the variants in order to more closely correlate the inability to negatively regulate activated receptors with absolute receptor number. In addition, transfections of endocytic targeting components, such as SNX-1, may be done in the parental cell lines to determine if an increase in the ability of these cells to traffic EGF-R's will decrease their tumorigenic phenotype.

The effect of covalent modification on S1046/1047 was not analyzed during this time period due to technical difficulties and it may still have a significant regulatory effect in these cells. For this reason these experiments are about to be performed.

Conclusions

1) Amplified receptor cell lines (468) do not seem to have the capacity to negatively regulate a large number of activated EGF-R's when compared to non-amplified variant cell lines (S1 and S11).

2) Neither the amplified parental cell line (468) nor the non-amplified receptor variants (S1 and S11) appear to be negatively regulated to any significant extent by PKC phosphorylation at T-654.

3) Non-amplified receptor variants (S1 and S11) are negatively regulated to a significant degree by tyrosine phosphatases though the amplified receptor parental cell lines do not appear to be regulated to any significant extent by tyrosine phosphatases.

4) The inability of the parental cell line to negatively regulate activated EGF-R's is largely due a lack in their ability to rapidly down-regulate the activated receptor.

5) The A1 cell line, which is phenotypicaly normal and possesses a receptor number greater than either of the non-amplified receptor variants (S1 or S11) but less than the receptor number of the amplified receptor cell line (468) is able to effectively decrease its level of activated receptors at a rate much greater than the 468 cell line.

Bibliography

1) Klijn, J.G.M., Berns, P.M.J.J., Schmitz, P.I.M., and Foekens, J.A. (1992) The Clinical Significance of Epidermal Growth Factor Receptor (EGF-R) in Human Breast Cancer: A review on 5232 Patients. *Endocr. Rev.* **13(1):**3-17.

2) Lund, K.A., and Wiley, H.S. (1993) Regulation of the Epidermal Growth Factor Receptor by Phosphorylation. in *Molecular Pharmacology of Cell Regulation.* V:**3.**(eds. D. Sibley and M. Houslay) John Wiley and Sons, Ltd., Sussex (1994).

3) Gill, G.N., Chen, W.S., Lazar, C.S., Glenney, J.R., Wiley, H.S., Ingraham, H.A., and Rosenfeld, M.G. (1988) Role of Intrinsic Protein Tyrosine Kinase in Function and Metabolism of the Epidermal Growth Factor Receptor. *Cold Spring Harb. Symp. Quant. Biol.* **LIII:**467-476.

4) Filmus, J., Pollak, M.N., Cailleau, R. and Buick, R.N. (1985). MDA-468, A Human Breast Cancer Cell Line with a High Number of Epidermal Growth Factor Receptors, has an Amplified EGF Receptor Gene and is Growth Inhibited By EGF. *Bioch. Bioph. Res. Comm.* **128(2):** 898-905

5) Filmus, J., Trent, J.M., Pollak, M.N., and Buick, R.N. (1987). Epidermal Growth Factor Gene-Amplified MDA-468 Breast Cancer Cell Line and Its Nonamplified Variants. *Mol. Cell. Biol.* **7(1):** 251-257.

6) Stampfer, M.R. and Bartley, J.C. (1985) Induction of Transformation and Continuous Cell Lines from Normal Human Mammary Epithelial Cells after Exposure to Benzo(a)pyrene. *Proc. Natl. Acad. Sci. USA* **82:**2394-2401.

7) Hammond, S.L., R.G., H., and Stampher, M.R. (1984) Serum-free Growth of Human Mammary Epithelial Cells: Rapid Clonal Growth in Defined Medium and Extended Serial Passage with Pituitary Extract. *Proc. Natl. Acad. Sci. USA.* **81:**5435-5441.

8) Walen, K. and Stampher, M.R. Chromosome Analysis of Human Mammary Epithelial Cells at Stages of Chemically-induced Transformation Progression to Immortality. *Cancer Genet. Cytogenet.* **37:**249-260.

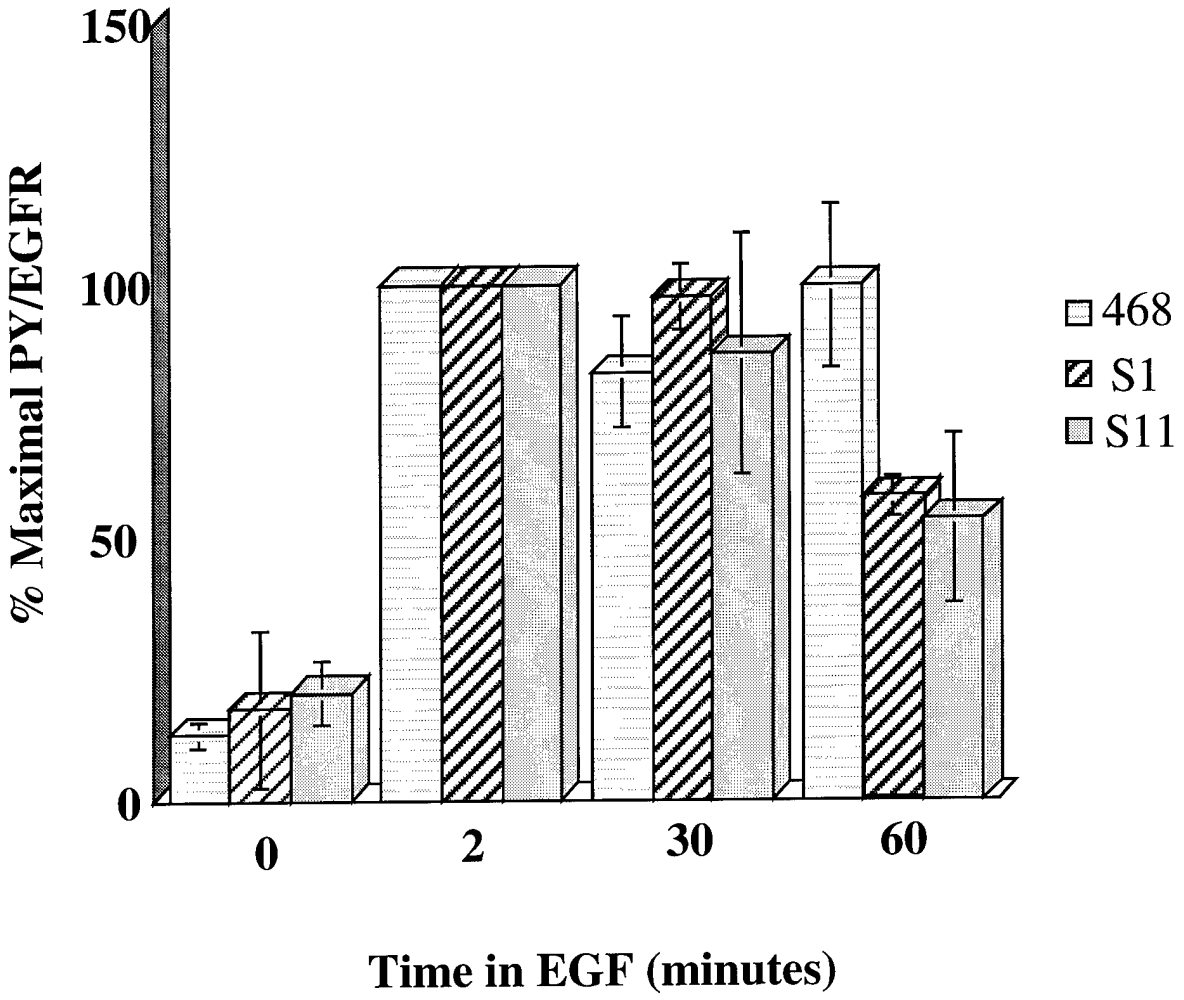

Figure 1: Receptor amplification prolongs EGF-R activation. 468 parental, S1, and S11 cell types were treated for 0, 2, 30 or 60 minutes with 100 ng/ml EGF. After protein isolation, PY/EGFR levels were analyzed by ELISA and normalized to 100% using the 2 minute timepoint as the maximum PY level.

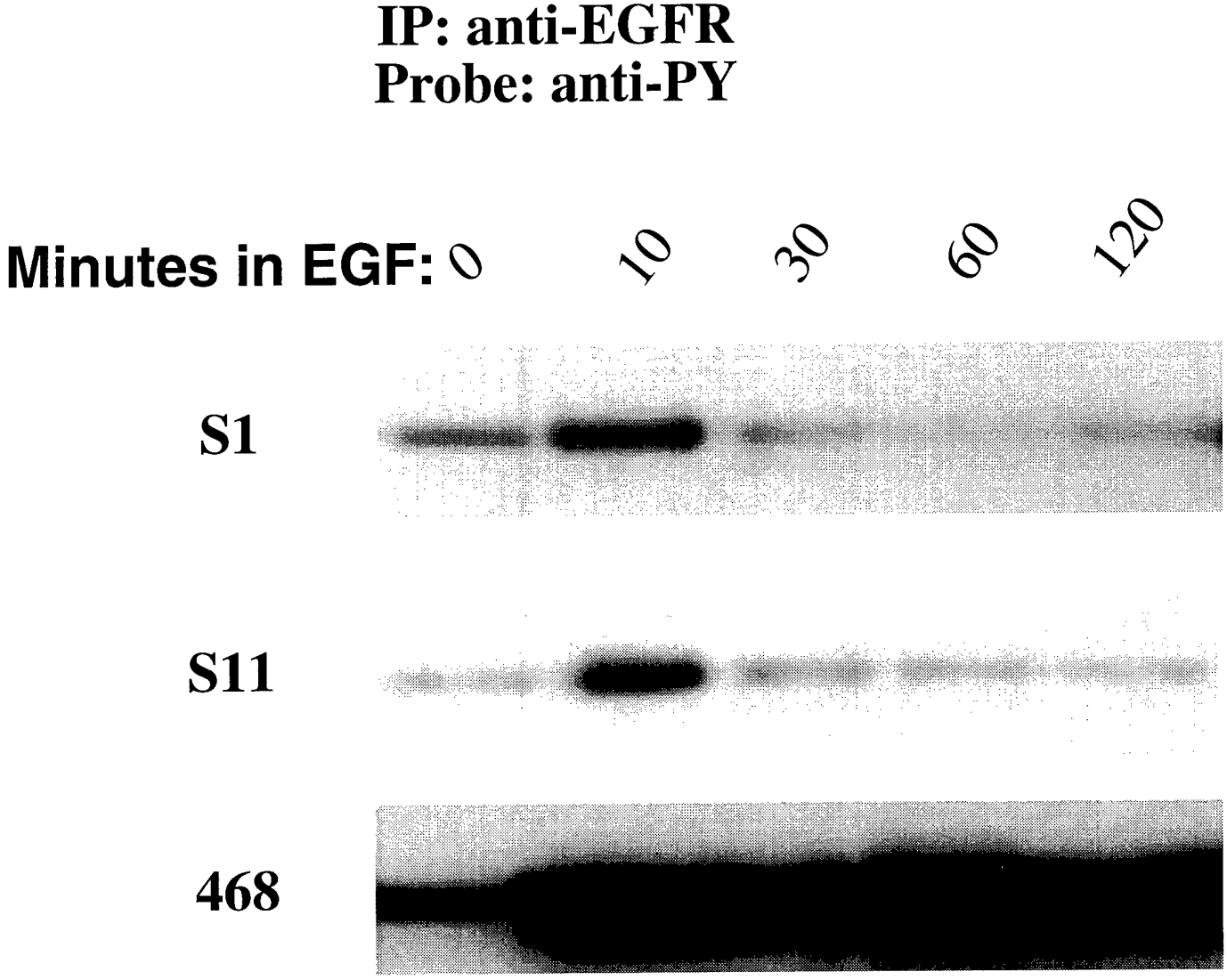

Figure 2: Total PY level is maintained at a maximum level in the parental cell type (468), but not in either of the variant cell types (S1 and S11). Cells were treated with 100 ng/ml EGF for 0, 10, 30, 60 or 120 minutes. Cellular protein was then extracted and EGF-R was immunoprecipitated. Protein bands were resolved by PAGE and visualized by westren blotting with anti-PY antibodies.

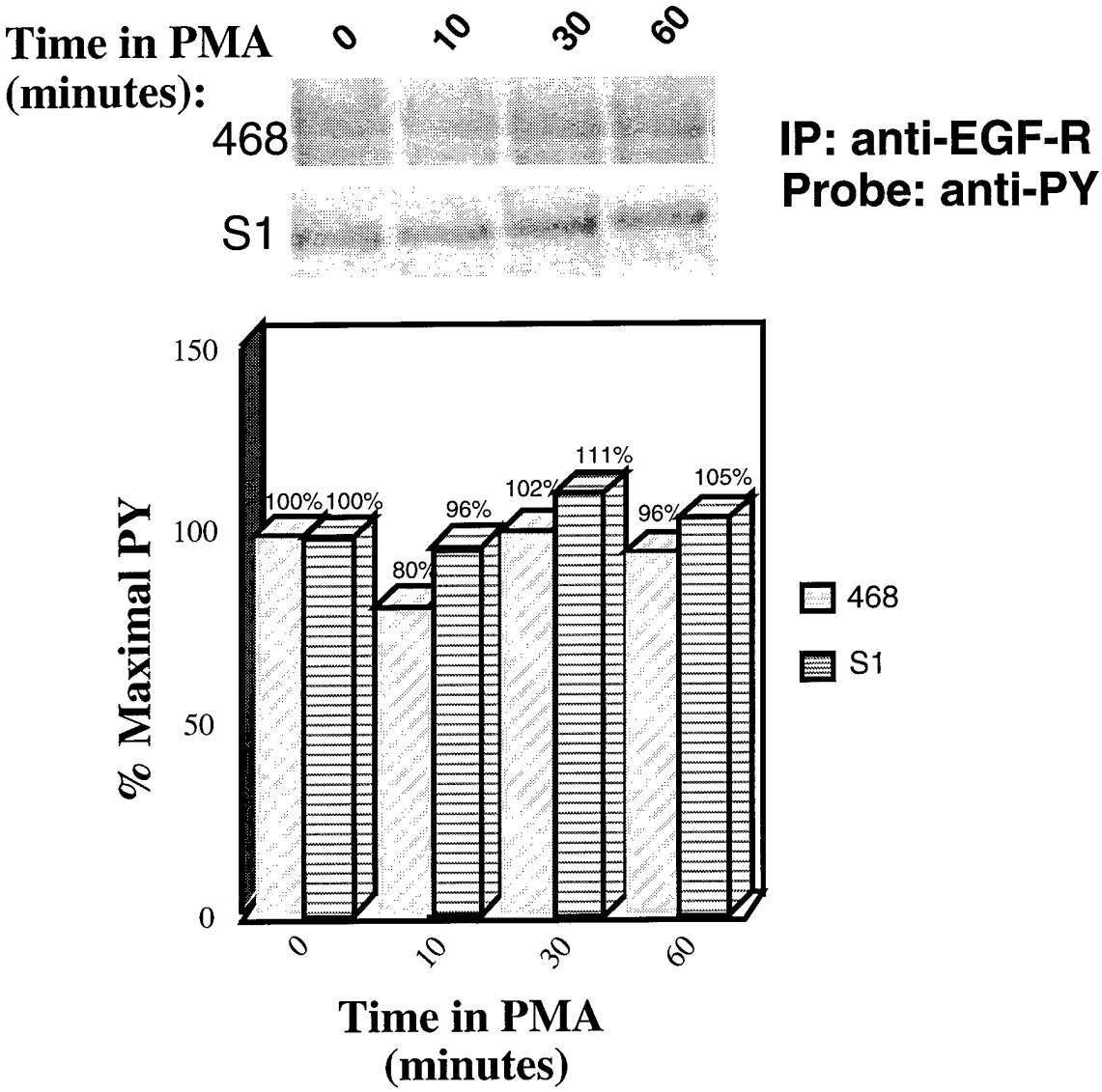

Figure 3: PMA treatment does not reduce the maximum level of PY in either the EGF-R amplified cell line or the non-amplified variant cell line (S1). Cells were treated for 0, 10, 30 or 60 minutes with 1.6 uM PMA and then treated for 10 minutes with 100 ng/ml EGF. Cellular proteins were extracted and the EGF-Rs were immunoprecipitated with anti-EGF-R antibody. The PY levels were visualizedby PAGE followed by western blotting with anti-PY antibodies. Values were normalized to 100% at 0 minutes treatment with PMA.

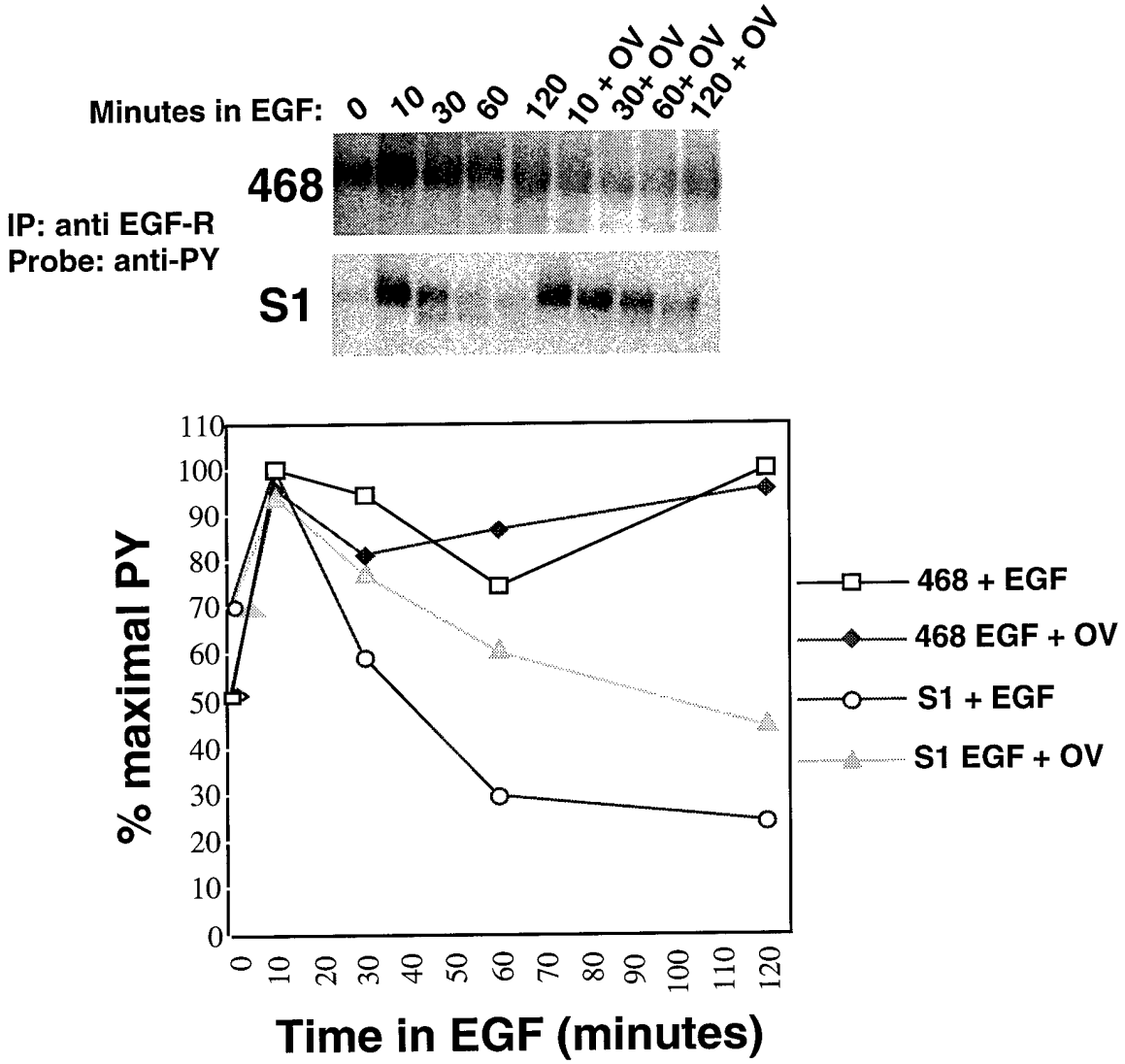

Figure 4: Cells were treated for 0, 10, 30, 60 or 120 minutes with 100 ng/ml EGF and + / - 1 m M orthovanadate. After the timecourse, the cellular proteins were extracted and the EGF-Rs were immunoprecipitated with anti-EGF-R antibodies. PY levels were visualized by western bloting for PY and all values were normalized to 100% at 10 minutes post-EGF treatment.

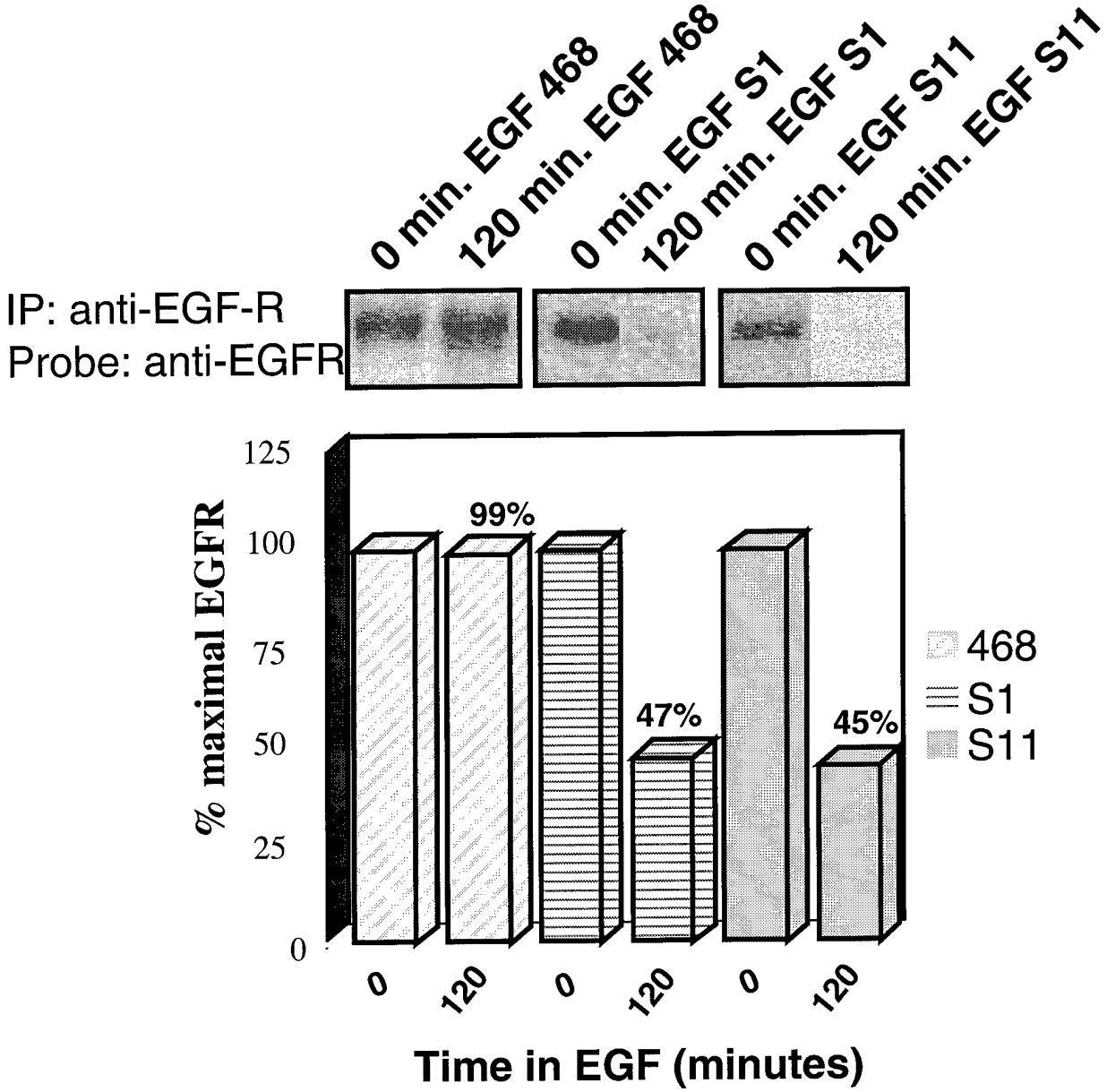

Figure 5: EGF-R's are not efficiently downregulated after 2 hours EGF treatment in amplified EGF-R cell lines but are efficiently downregulated in the non-amplified variants. Cells were treated from 0 to 120 minutes with 100 ng/ml EGF. After incubation, cellular proteins were extracted and the EGF-R was immunoprecipitated with anti-EGF-R antibodies. The bands were visualized by western blotting with a different anti-EGF-R antibody. samples were normalized by setting the 0 minutes timepoint to 100%.

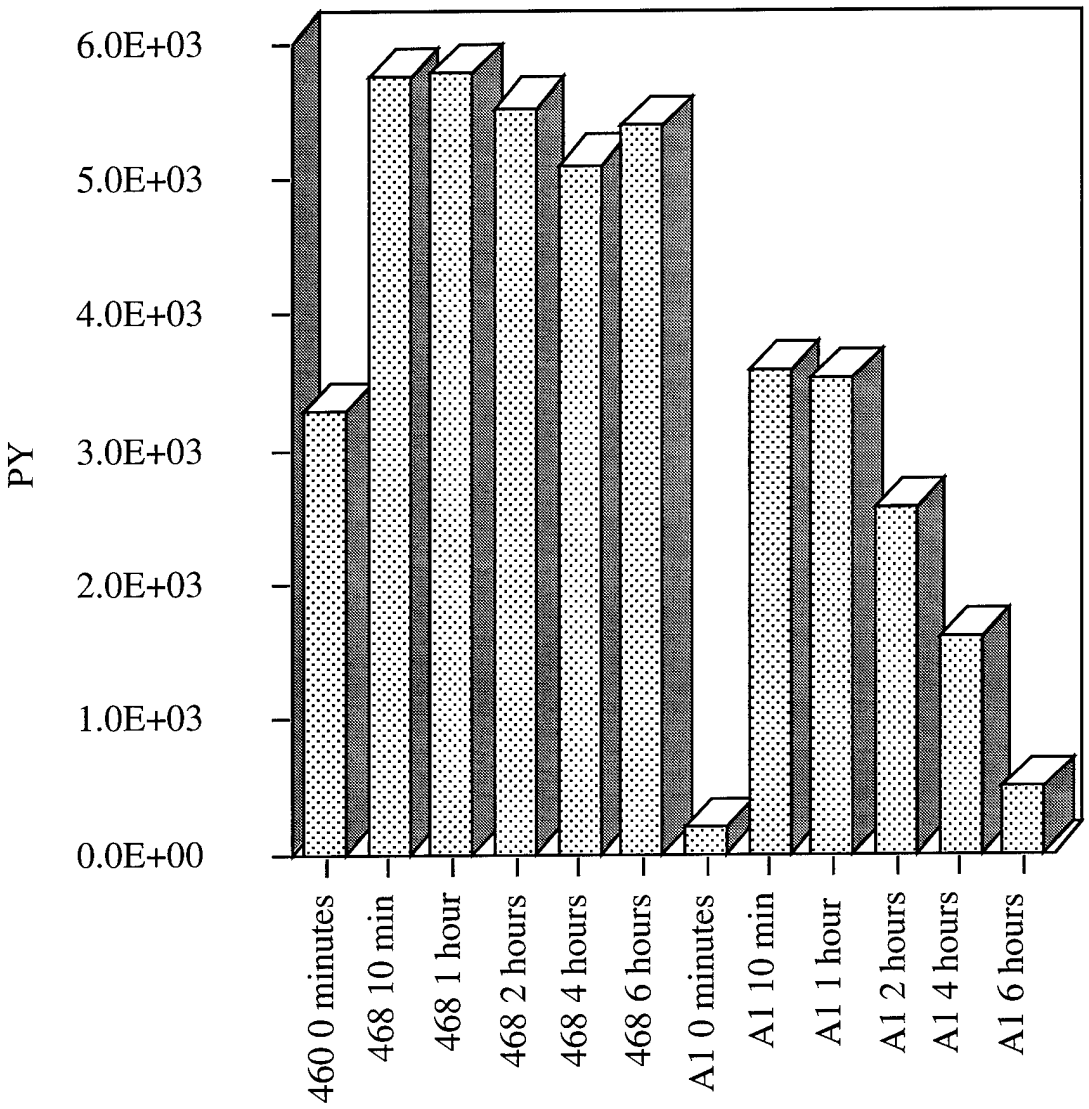

Figure 6: A1 reduces its number of activated receptors at a rate intermediate to the unamplified variants and the amplified parental cell type. Cells were treated for 0 , 10 minutes, 1 hour, 2 hours, 4 hours or 6 hours with 100 ng/ml EGF. After incubation the cellular proteins were isolated and EGF-Rs were isolated by immunoprecipitation with anti-EGF-R antibodies. PY was analyzed by western blotting with anti-PY antibody.